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Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier

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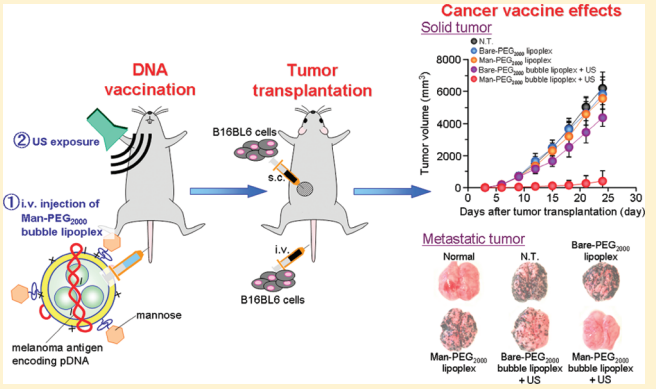
Supporting Information

ABSTRACT: DNA vaccination has attracted much attention as a promising therapy for the prevention of metastasis and relapse of malignant tumors, especially highly metastatic tumors such as melanoma. However, it is difficult to achieve a potent cancer vaccine effect by DNA vaccination, since the number of dendritic cells, which are the major targeted cells of DNA vaccination, is very few. Here, we developed a DNA vaccination for metastatic and relapsed melanoma by ultrasound (US)-responsive and antigen presenting cell (APC)-selective gene carriers reported previously, named Man-PEG$_{2000}$ bubble lipoplexes. Following immunization using US exposure and Man-PEG$_{2000}$ bubble lipoplexes constructed with pUb-M, which expresses ubiquitylated melanoma-specific antigens (gp100 and TRP-2), the secretion of Th1 cytokines (IFN-γ and TNF-α) and the activities of cytotoxic T lymphocytes (CTLs) were specifically enhanced in the presence of B16BL6 melanoma antigens. Moreover, we succeeded in obtaining potent and sustained DNA vaccine effects against solid and metastatic tumor derived from B16BL6 melanoma specifically. The findings obtained from this study suggest that the gene transfection method using Man-PEG$_{2000}$ bubble lipoplexes and US exposure could be suitable for DNA vaccination aimed at the prevention of metastatic and relapsed cancer.



KEYWORDS: mannose modification, bubble lipoplex, ultrasound, DNA vaccination, melanoma

INTRODUCTION

Melanoma is a neoplasm arising within epidermal melanocytes of the skin, and one of several cancers exhibiting the increasing incidence in recent years.[1] Early stage melanoma is curable, but melanoma metastasis and relapse occur frequently in the patients following treatments such as surgery, and the prognosis for patients with metastatic melanoma is poor.[2,3] Although systemic therapy induces complete therapeutic responses in a minority of patients, metastatic melanoma is a devastating illness and treatment options are limited; therefore, there is a need for an effective therapy for metastatic melanoma.

Cancer vaccination has attracted much attention as a promising therapy for the prevention of tumor growth and metastasis, because it is based on an immune response provided by the cancer antigen, and consequently, its therapeutic effects are specific to the targeted cancer cells and the adverse effects followed by cancer vaccination are low.[4,5] In particular, it has been reported that DNA vaccination, which uses an exogenous gene encoding cancer antigen, can induce not only humoral immunity but also cellular immunity and, moreover, can induce cancer-specific CTLs with potent antitumor activities.[6−9] In a variety of cancers, since melanoma is known to exhibit inherent immunogenicity and the identification of melanoma-specific antigen is proceeding, such as gp100, melanoma-antigen recognized by T cells-1 (MART-1) and tyrosinase-related protein (TRP),[10−13] it is considered that DNA vaccination against melanoma is suitable for not only the

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prevention of metastasis and relapse but also the suppression of tumor growth.

To achieve potent therapeutic effects by DNA vaccination against cancer, it is essential to transfer the antigen-coding gene selectively and efficiently into APCs such as macrophages and dendritic cells, which play a pivotal role in the initiation, programming and regulation of cancer-specific immune responses.[14,15] Our group has also developed mannose-modified liposome/plasmid DNA (pDNA) complexes (mannose-modified lipoplexes) for APC-selective gene transfer via mannose receptors expressing on APCs, and obtained APC-selective gene expression in the liver and spleen by mannose-modified lipoplexes.[16,17] Moreover, our group also succeeded in obtaining DNA vaccine effects against cancer by intraperitoneal administration of mannose-modified lipoplexes constructed with tumor-specific antigen coding pDNA, such as ovalbumin (OVA) and melanoma-related antigens.[18,19] However, the gene transfection efficiency into APCs was lower than that in other cells;[20] therefore, it could be difficult to induce a potent cancer vaccine effect for the prevention of metastasis and relapse by DNA vaccination using conventional lipofection methods.

It has been reported that cancer vaccine effects can be enhanced by physical stimulation-mediated gene transfer such as electroporation,[21,22] hydrodynamic injection[23,24] and sonoporation methods.[25] These transfection methods enable the delivery of a large amount of antigen-coding gene and antigen peptides into APCs, since exogenous materials are directly introduced into the cytoplasm without endocytosis in these methods.[26−29] Recently, we have applied "sonoporation methods[25,29−31]" using US exposure and microbubbles enclosing US imaging gas to enhance gene expression in APCs[32] and developed a gene transfection method for DNA vaccination using US-responsive and mannose-modified gene carriers, Man-PEG_{2000} bubble lipoplexes.[33] This method enables APC-selective and -efficient gene expression, and moreover, effective vaccine effects against OVA-expressing cancer cells were obtained by applying this method to DNA vaccination using OVA-encoding pDNA.[33] However, the antigenicity of OVA is extremely high compared with other antigens,[34] and it is difficult to extrapolate the result obtained by DNA vaccination against OVA-expressing cells to actual cancer therapy, since OVA-expressing cells are transfectant constructed by gene transfer. Therefore, it is unclear if DNA vaccination by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure is effective against cancer, i.e. melanoma, with metastatic properties.

In this study, we examined DNA vaccine effects against melanoma by transfection of pUb-M, coexpressing ubiquitylated gp100 and TRP-2, using Man-PEG_{2000} bubble lipoplexes and US exposure. First, we examined the level of gene expression in the splenic dendritic cells by gene transfer using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. Second, we studied the characteristics of cytokine secretion and the induction of CTL activities against B16BL6 cell-derived melanoma by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. Then, we investigated the cancer vaccine effects against solid and metastatic tumors derived from B16BL6 cells by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Finally, we evaluated the duration of cancer vaccine effects against solid and metastatic melanoma after pUb-M transfection using Man-PEG_{2000} bubble lipoplexes and US exposure.

EXPERIMENTAL SECTION

Materials. 1,2-Stearoyl-3-trimethylammoniumpropane (DSTAP), 1,2-distearoyl-*sn*-glycero- 3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-2000] (NH_2-PEG_{2000}-DSPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), Sigma Chemicals Inc. (St. Louis, MO, USA) and NOF Co. (Tokyo, Japan), respectively. Anti-CD11c monoclonal antibody (N418)-labeled magnetic beads were obtained from Miltenyi Biotec Inc. (Auburn, CA, USA). Fetal bovine serum (FBS) was purchased from Equitech-bio Inc. (Kerrville, TX, USA). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

pDNA, Cell Lines and Mice. pUb-M containing murine melanoma glycoprotein-100_{25-33} (gp100) and tyrosinase-related protein-$2_{181-188}$ (TRP-2) peptide epitopes was kindly provided by Prof. R. A. Reisfeld.[35] The B16BL6 melanoma cells, colon-26 adenocarcinoma cells and EL4 lymphoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The B16BL6/Luc cells and colon-26/Luc cells, which are cell lines expressing firefly luciferase stably, were established as previously reported.[36,37] The B16BL6 cells and EL4 cells were cultured in DMEM, and the colon-26 cells were cultured in RPMI-1640 at 37 °C in 5% CO_2. Both media were supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Female C57BL/6 mice (6 weeks old) and female Balb/c mice (6 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

Construction of Man-PEG_{2000} Bubble Lipoplexes. Man-PEG_{2000} bubble lipoplexes were constructed according to our previous report.[33] Briefly, DSTAP, DSPC and NH_2-PEG_{2000}-DSPE or mannose-modified PEG_{2000}-DSPE were mixed in chloroform at a molar ratio of 7:2:1 to produce the liposomes for bubble lipoplexes. The mixture for construction of liposomes was dried by evaporation and vacuum desiccated, and the resultant lipid film was resuspended in sterile 5% dextrose. After hydration for 30 min at 65 °C, the dispersion was sonicated for 10 min in a bath sonicator and for 3 min in a tip sonicator to produce liposomes. Then, the liposomes were sterilized by passage through a 0.45 μm filter (Nihon-Millipore, Tokyo, Japan). The lipoplexes were prepared by gently mixing equal volumes of pDNA and liposome solution at a charge ratio of 1.0:2.3 (−:+). To enclose US imaging gas in lipoplexes, the prepared lipoplexes were pressured with perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan) and sonicated using a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min. The particle sizes and zeta potentials of the liposomes/lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern Instrument, Ltd., Worcestershire, U.K.).

In Vivo Gene Transfection Method. Six week old C57BL/6 female mice were intravenously injected with 400 μL of bubble lipoplexes via the tail vein using a 26 gauge syringe needle at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate,

Table 1. Particle Sizes and Zeta Potentials of Lipoplexes and Bubble Lipoplexes Constructed with pUb-M[a]

	particle size (nm)	zeta-potential (mV)
Bare-PEG_{2000} lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE = 7:2:1 (mol))	144 ± 13	45.7 ± 4.5
Man-PEG_{2000} lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE = 7:2:1 (mol))	143 ± 10	44.5 ± 5.8
Bare-PEG_{2000} bubble lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE = 7:2:1 (mol))	557 ± 20	46.7 ± 4.2
Man-PEG_{2000} bubble lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE = 7:2:1 (mol))	555 ± 19	45.1 ± 2.2

[a] Each value represents the mean ± SD (n = 3).

10 Hz; intensity 1.0 W/cm^2; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator with a probe of diameter 20 mm. At predetermined times after injection, mice were sacrificed and spleens were collected for each experiment. In the intradermal transfection study, mice were intradermally injected with 200 μL of bubble lipoplexes at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2; time, 2 min) was directly exposed to the injected site using a probe of diameter 6 mm. In the intrasplenic transfection, mice were directly injected into the spleen with 200 μL of bubble lipoplexes at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2; time, 2 min) was directly exposed to the spleen using a probe of diameter 6 mm.

Measurement of the Level of mRNA Expression. Total RNA was isolated from the spleen using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of mRNA was carried out using a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). The detection of the Ub-M cDNA was carried out by real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, USA) with primers. The primers for Ub-M, gp100, TRP-2 and GAPDH cDNA were constructed as follows: primer for Ub-M cDNA, 5′-GAG CCC AGT GAC ACC ATA GA-3′ (forward) and 5′-GTG CAG GGT GGA CTC TTC CT-3′ (reverse); primer for gp100, 5′-GCA CCC AAC TTG TTG TTC CT-3′ (forward) and 5′-GTG CTA CCA TGT GGC ATT TG-3′ (reverse); primer for TRP-2, 5′-CTT CCT AAC CGC AGA GCA AC-3′ (forward) and 5′-CAG GTA GGA GCA TGC TAG GC-3′ (reverse); primer for GAPDH, 5′-TCT CCT GCG ACT TCA ACA-3′ (forward) and 5′-GCT GTA GCC GTA TTC ATT GT-3′ (reverse) (Sigma-Aldrich, St. Louis, MO, USA). The mRNA copy numbers were calculated for each sample from the standard curve using the instrument software ("Arithmetic Fit Point analysis" for the Lightcycler). Results were expressed as relative copy numbers calculated relative to GAPDH mRNA (copy numbers of Ub-M, gp100 and TRP-2 mRNA/copy numbers of GAPDH mRNA).

Isolation of Splenic $CD11c^+$ Cells (Dendritic Cells) in Mice. At 6 h after transfection, spleens were harvested and spleen cells were suspended in ice-cold RPMI-1640 medium on ice. Then, red blood cells were removed by incubation with hemolytic reagent (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 3 min at room temperature. $CD11c^+$ and $CD11c^-$ cells were separated by magnetic cell sorting with an auto MACS (Miltenyi Biotec Inc., Auburn, CA, USA) following the manufacturer's instructions.

Evaluation of Antigen-Specific Cytokine Secretion. To prepare the tumor cell lysates (B16BL6 cells, EL4 cells and colon-26 cells), the cells were scraped from the plates and suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). After three cycles of freezing and thawing, the lysates were centrifuged at 10000g, 4 °C for 10 min and the resultant supernatants were collected. The protein concentration of cell lysates was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 96-well plates and incubated for 72 h at 37 °C in the presence or absence of tumor cell lysates (100 μg of proteins). IFN-γ, TNF-α, IL-4 and IL-6 in the culture medium were measured using a suitable commercial ELISA Kit (Bay Bioscience Co., Ltd., Hyogo, Japan).

CTL Assay. At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 6-well plates and coincubated with mitomycin C-treated tumor cells (B16BL6 cells, EL4 cells and colon-26 cells) for 4 days. After 4 days of coincubation, nonadherent cells were harvested, washed and plated in 96-well plates with target cells (B16BL6 cells, EL4 cells and colon-26 cells) at various effector cell/target cell (E/T) ratios. The target tumor cells were labeled with ^{51}Cr by incubating with $Na_2{}^{51}CrO_4$ (PerkinElmer, Inc., MA, USA) in culture medium for 1 h at 37 °C. At 4 h after incubation, the plates were centrifuged, and the supernatant in each well was collected and the radioactivity of released ^{51}Cr was measured in a gamma counter. The percentage of ^{51}Cr release was calculated as follows: specific lysis (%) = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100).

Therapeutic Experiments in Solid Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells, EL4 cells and colon-26 cells were transplanted subcutaneously into the back of the mice (1×10^6 cells). The tumor size was measured with calipers in two dimensions, and the tumor volume was calculated using the following equation: volume (mm^3) = $\pi/6 \times$ longer diameter × (shorter diameter)^2. The survival of the mice was monitored up to 100 days after the transplantation of tumor cells.

Therapeutic Experiments in Lung Metastatic Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells or colon-26 cells were intravenously administered via the tail vein (1×10^5 cells) and the survival of the mice was monitored up to 100 days after administration of the tumor cells. To evaluate metastasis, B16BL6/Luc cells or colon-26/Luc cells were intravenously administered via the tail vein (1×10^5 cells). At 14 days after the administration of the tumor cells, the number of B16BL6/Luc cells and colon-26/Luc cells in the lung was quantitatively evaluated by measuring luciferase activity as previously reported.[36,37]

Statistical Analysis. Results were presented as the mean ± SD of more than three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Two-group comparisons were performed by the Student's t test. Multiple comparisons between control groups and other groups were performed by the Dunnett's test, and multiple

comparisons between all groups were performed by the Tukey–Kramer test.

RESULTS

Physicochemical Properties of Bubble Lipoplexes Constructed with pUb-M. The physicochemical properties of lipoplexes and bubble lipoplexes constructed with pUb-M used in all experiments were evaluated by measuring the particle sizes and zeta potentials. The mean particle sizes and zeta potentials of nonmodified PEG_{2000}-lipoplexes (Bare-PEG_{2000} lipoplexes) and mannose-conjugated PEG_{2000}-lipoplexes (Man-PEG_{2000} lipoplexes) were 144 ± 13 nm, 45.7 ± 4.5 mV and 143 ± 10 nm, 44.5 ± 5.8 mV, respectively (Table 1). Moreover, the mean particle sizes and zeta potentials of nonmodified bubble lipoplexes (Bare-PEG_{2000} bubble lipoplexes) and Man-PEG_{2000} bubble lipoplexes were 557 ± 20 nm, 46.7 ± 4.2 mV and 555 ± 19 nm, 45.1 ± 2.2 mV, respectively (Table 1). These results corresponded to our previous reports using other pDNA,[33] suggesting that pDNA had no effect on the physicochemical properties of Man-PEG_{2000} bubble lipoplexes.

Splenic Dendritic Cell-Selective and -Efficient Gene Expression by Gene Transfer Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. First, to investigate the level of gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure in the spleen, we measured the relative mRNA copy numbers of Ub-M after transfection. As shown in Figures 1A and 1B, the level of Ub-M mRNA expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure reached a peak at 6 h after transfection. Moreover, that level of Ub-M mRNA expression was markedly higher than that obtained by Bare- and Man-PEG_{2000} lipoplexes, and significantly higher than that obtained by Bare-PEG_{2000} bubble lipoplexes and US exposure. Then, we investigated the mannose receptor-expressing cell selectivity of Ub-M mRNA expression obtained by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure. In the spleen, the relative mRNA copy numbers of Ub-M in $CD11c^+$ cells was significantly higher than that in $CD11c^-$ cells following transfection using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 1C). On the other hand, no selective gene expression in $CD11c^+$ cells was observed by gene transfer using Bare-PEG_{2000} bubble lipoplexes and US exposure (Figure 1C).

Antigen-Stimulatory Th1 Cytokine Secretion from the Splenic Cells Immunized by Man-PEG_{2000} Bubble Lipoplexes and US Exposure. To evaluate the melanoma-specific cytokine secretion from immunized splenic cells, splenic cells immunized by pUb-M were incubated with each tumor cell-lysate in vitro, and then, Th1 and Th2 cytokines secreted in the supernatants were measured. Following investigation of the expression level of gp100 and TRP-2, a melanoma-specific antigen, in each cell used in this study, the expression of gp100 and TRP-2 was only detected in B16BL6 cells which are melanoma cell lines (Supplementary Figure 1 in the Supporting Information). As results of the immunization according to the protocol shown in Figure 2A, the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes and US exposure secreted the highest amount of IFN-γ and TNF-α, which are Th1 cytokines, in the presence of B16BL6 cell lysates (Figures 2B and 2C). On the other hand, the secretion of Th1 cytokines (IFN-γ and TNF-α) was lower in all the groups in the presence of EL4 and colon-26 cell lysates. Moreover, the secretion of IL-4 and IL-6, which are Th2 cytokines, was also lower in all the groups in the presence of each cell lysate (Figures 2D and 2E). These observations suggest that pUb-M transfer by Man-PEG_{2000} bubble lipoplexes and US exposure significantly enhances the differentiation of helper T cells into Th1.

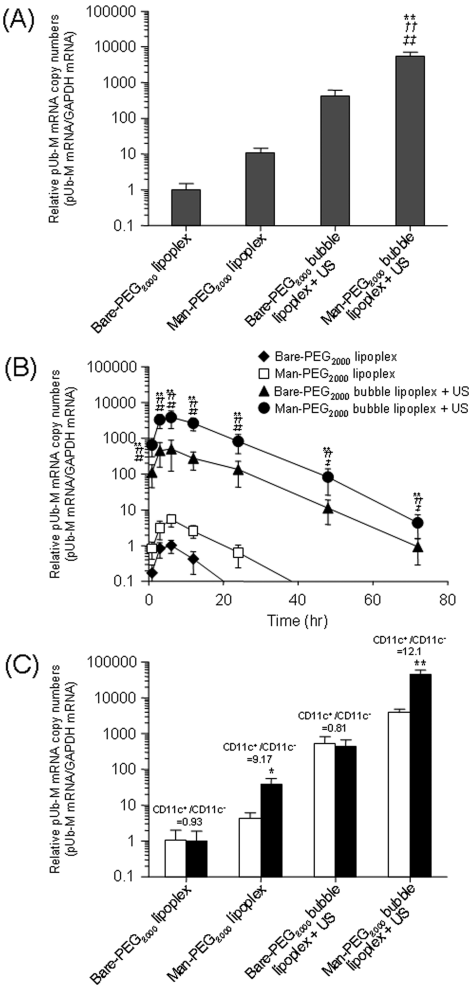

Figure 1. Enhanced Ub-M mRNA expression in the spleen and the splenic dendritic cells ($CD11c^+$ cells) by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure in vivo. (A) The level of Ub-M mRNA expression obtained by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA) in the spleen at 6 h after transfection. Each value represents the mean + SD (n = 4). $^{**}p < 0.01$, compared with Bare-PEG_{2000} lipoplex; $^{\dagger\dagger}p < 0.01$, compared with Man-$PEG_{2000}$ lipoplex; $^{\ddagger\ddagger}p < 0.01$, compared with Bare-$PEG_{2000}$ bubble lipoplex + US. (B) Time-course of Ub-M mRNA expression in the spleen after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). Each value represents the mean ± SD (n = 4). $^{**}p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} lipoplex; $^{\dagger\dagger}p < 0.01$, compared with the corresponding group of Man-PEG_{2000} lipoplex; $^{\ddagger}p < 0.05$; $^{\ddagger\ddagger}p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} bubble lipoplex + US. (C) Splenic cellular localization of Ub-M mRNA expression at 6 h after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). Each value represents the mean + SD (n = 4). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of $CD11c^-$ cells.

Induction of Melanoma-Specific CTLs by pUb-M Transfer Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. We investigated the melanoma-specific CTL activities in the

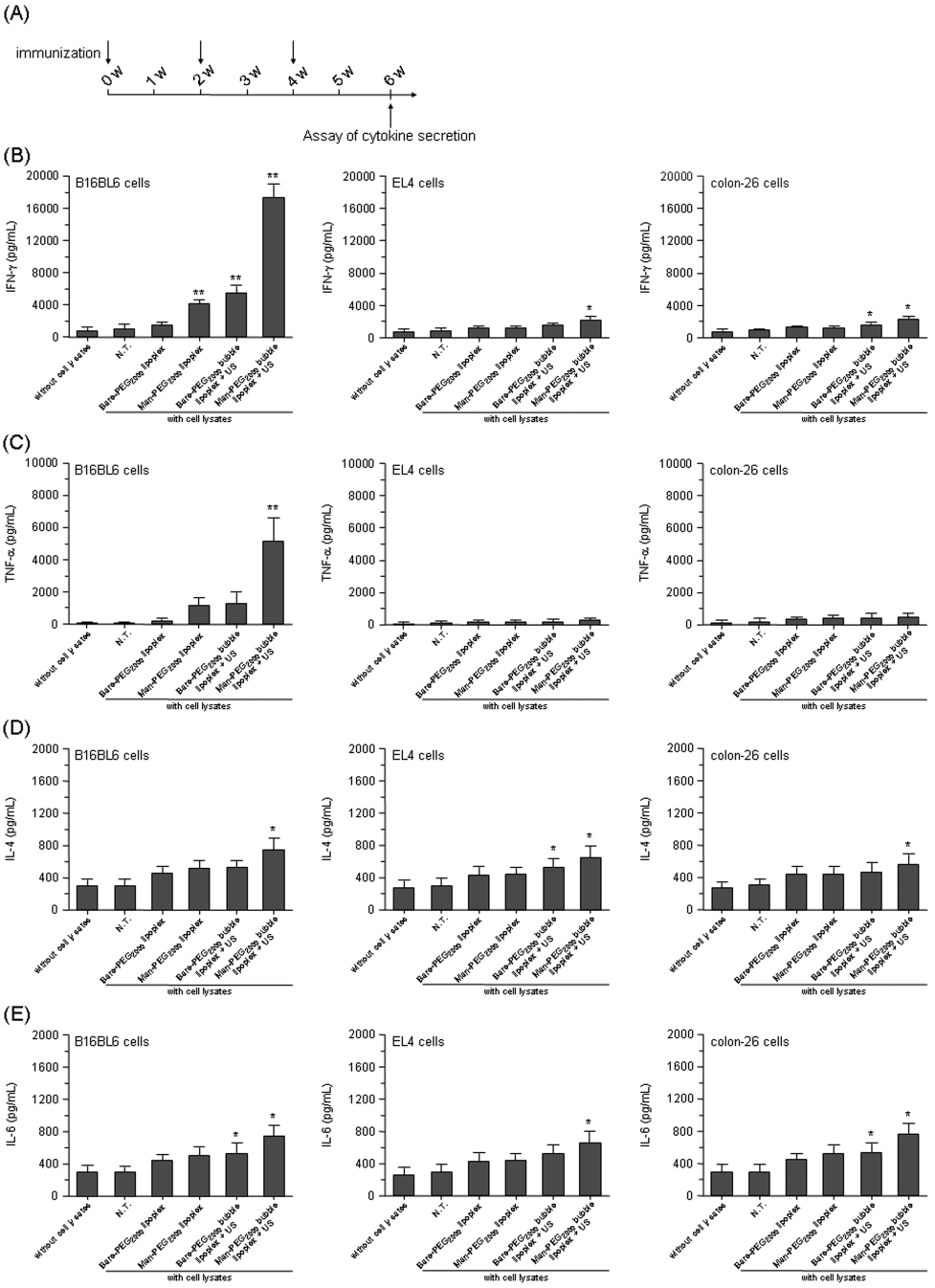

Figure 2. Melanoma-stimulatory cytokine secretion characteristics by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. (A) Schedule of immunization for the evaluation of melanoma-stimulatory cytokine secretion characteristics. (B−E) Each cancer cell lysate-specific IFN-γ (B), TNF-α (C), IL-4 (D) and IL-6 (E) secretion from the splenic cells immunized three times biweekly with Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The splenic cells were collected at 2 weeks after the last immunization. After the immunized splenic cells were cultured for 72 h in the presence of each cancer cell lysate (100 μg protein), IFN-γ, TNF-α, IL-4 and IL-6 secreted in the medium were measured by ELISA. Each value represents the mean + SD (n = 4). *p < 0.05; **p < 0.01, compared with the corresponding "without cell lysate" group.

splenic cells immunized by pUb-M. This experiment was performed according to the protocol shown in Figure 3A. As shown in Figure 3B, the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes and US exposure showed the highest CTL activities of all groups stimulated by B16BL6 cells. In contrast, no CTL activity was obtained in all groups stimulated by EL4 and colon-26 cells (Figures 3C and 3D). These results suggest that melanoma-specific CTLs are induced effectively in the splenic cells transfected pUb-M by Man-PEG_{2000} bubble lipoplexes and US exposure.

Cancer Vaccine Effects against Melanoma-Derived Solid and Metastatic Tumors by DNA Vaccination Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. Cancer vaccine effects against solid and metastatic tumors obtained by DNA vaccination using Man-PEG_{2000} lipoplexes and US exposure were examined. First, we evaluated the level of gp100 and

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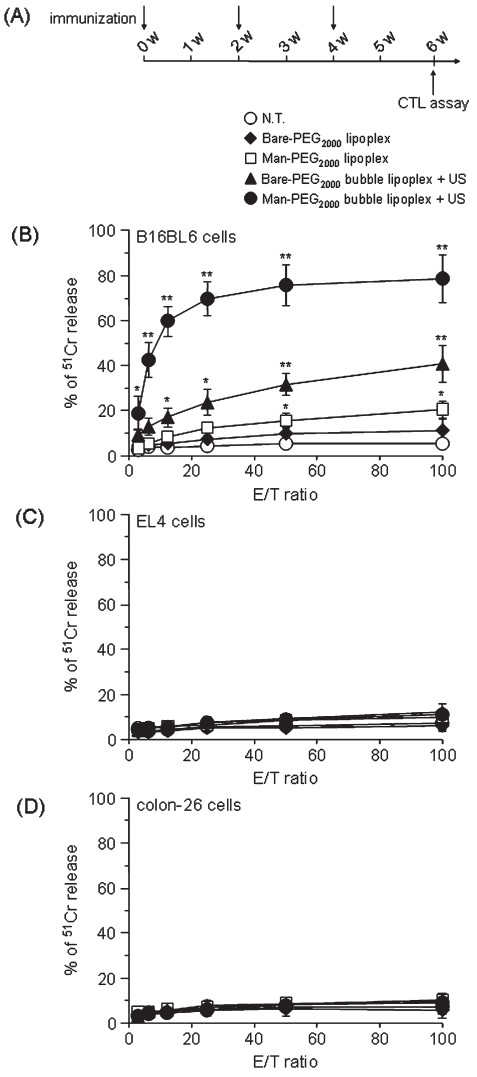

Figure 3. Evaluation of melanoma-specific CTL activities by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of immunization for the assay of melanoma-specific CTL activities. (B−D) Each cancer cell lysate-specific CTL activities after immunization three times with Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The splenic cells were collected at 2 weeks after the last immunization, and then, the splenic cells were coincubated with ^{51}Cr-labeled cancer cells. CTL activities against B16BL6 cells (B), EL4 cells (C) and colon-26 cells (D) in the immunized splenic cells were determined by ^{51}Cr release assay. Each value represents the mean ± SD (n = 4). *p < 0.05; **p < 0.01, compared with the corresponding "N.T." (no treatment) group.

TRP-2 expression in each tumor used in this study, and confirmed that the expression of gp100 and TRP-2 was only detected in B16BL6 tumor (Supplementary Figure 2 in the Supporting Information). Following investigation of cancer vaccine effects against solid tumors according to the protocol shown in Figure 4A, B16BL6-transplanted tumor growth was significantly suppressed in mice immunized with Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure (Figures 4B and 4D). Moreover, the survival of B16BL6-transplanted mice was significantly prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure, and complete tumor-rejection was observed in 7/10 of B16BL6-transplanted mice (Figure 4C). These vaccine effects were obtained against B16F1-transplanted mice (Supplementary Figure 4B in the Supporting Information); on the other hand, no cancer vaccine effects against EL4 and colon-26 cell-derived tumors, which do not express gp100 and TRP-2, were observed in all groups (Figures 4B−D). In addition, these DNA vaccine effects against B16BL6-derived tumors were not observed in mice immunized by Man-PEG_{2000} bubble lipoplexes constructed with pcDNA3.1 (control vector) and US exposure (Supplementary Figure 3 in the Supporting Information), suggesting that DNA vaccine effects against melanoma are attributed to not pDNA transfer itself but melanoma-related antigens expressed by pUb-M.

Then, we investigated the cancer vaccine effects against a pulmonary metastatic tumor obtained by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Figure 5A, the level of luciferase expression derived from B16BL6/Luc cells in the lung, which express gp100 and TRP-2 (Supplementary Figures 1 and 2 in the Supporting Information), was significantly suppressed in mice immunized by Man-PEG_{2000} bubble lipoplexes and US exposure (Figures 5B and 5D). Moreover, the survival of the pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 5C). These vaccine effects were obtained against pulmonary metastatic B16F1-derived tumor model mice (Supplementary Figure 4C in the Supporting Information); on the other hand, no therapeutic effects against colon-26 cells by DNA vaccination using this method were observed in any of the groups (Figures 5B−D).

Effect of Administration Routes of Man-PEG_{2000} Bubble Lipoplexes on Cancer Vaccine Effects. Next, we evaluated the effects of the administration routes of Man-PEG_{2000} bubble lipoplexes to obtain effective DNA vaccine effects. In this experiment, in addition to pUb-M transfer using intravenous administration of Man-PEG_{2000} bubble lipoplexes and external US exposure, we investigated the DNA vaccine effects by pUb-M transfer using intradermal and intrasplenic administration of Man-PEG_{2000} bubble lipoplexes and direct US exposure to the administration sites. In the preliminary experiments about US intensity for obtaining the highest gene expression in the spleen and skin, the optimized intensities of US exposure to the abdominal area by a probe of diameter 20 mm and to the injected sites directly by a probe of diameter 6 mm are 1.0 W/cm^2 and 4.0 W/cm^2, respectively (data not shown). Based on these investigations, we used the different US intensity depending on the probe size and US-exposed sites in this study. Following immunization against melanoma according to the protocol shown in Figure 6A, B16BL6-transplanted tumor growth was suppressed the best in mice transfected with pUb-M using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figure 6B). Moreover, the survival of B16BL6-transplanted mice was also prolonged the best by DNA vaccination using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figure 6C).

Duration of DNA Vaccine Effects by Man-PEG_{2000} Bubble Lipoplexes and US Exposure. Finally, to investigate the duration of DNA vaccine effects following pUb-M transfer using Man-PEG_{2000} bubble lipoplexes and US exposure, B16BL6 cells were retransplanted in mice in which first-transplanted tumors derived from B16BL6 cells were completely rejected by DNA

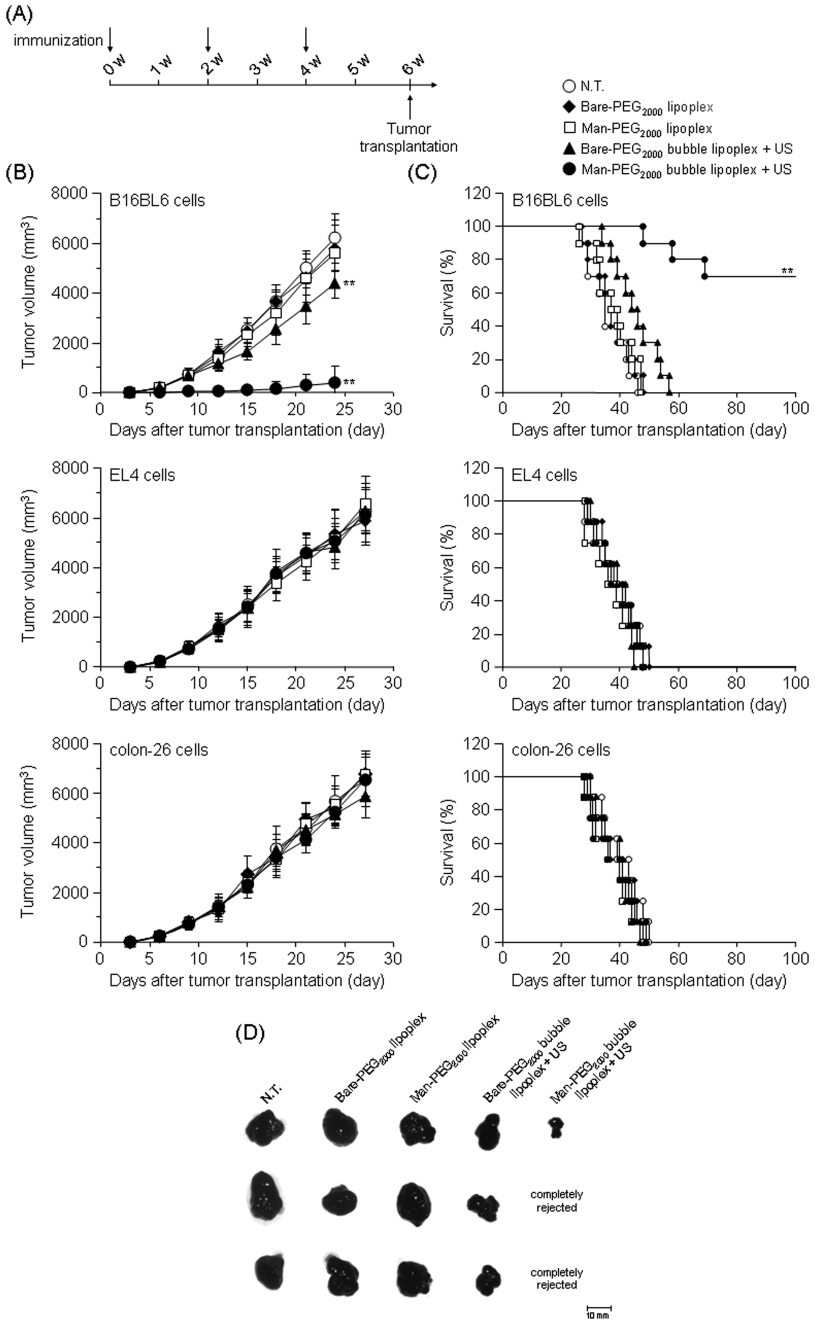

Figure 4. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments on solid tumors. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging effects of survival in tumor-transplanted mice (C) by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA). Two weeks after the last immunization, B16BL6 cells, EL4 cells and colon-26 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice (n = 8−10). The tumor volume was evaluated (each value represents the mean ± SD), and the survival was monitored up to 100 days after the tumor transplantation. $^{**}p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6 cell-derived solid tumor at 15 days after the tumor transplantation in mice immunized by each transfection method (n = 3).

vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure at 100 days after the first transplantation (Figure 7A). As shown in Figure 7B, compared with N.T. mice, the second-transplanted tumor growth derived from B16BL6 cells was markedly suppressed and the survival of B16BL6-transplanted mice was significantly prolonged. In addition, we also evaluated the duration of DNA vaccine effects against a pulmonary metastatic tumor. Following intravenous injection of B16BL6/Luc cells into mice at 100 days after the last immunization (Figure 7C), the level of luciferase expression derived from B16BL6/Luc cells in the lung was significantly suppressed and the survival of pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged in mice transfected with pUb-M using Man-PEG$_{2000}$ bubble lipoplexes and US exposure (Figure 7D). These results suggest that DNA vaccine effects by pUb-M transfer using Man-PEG$_{2000}$ bubble lipoplexes and US exposure

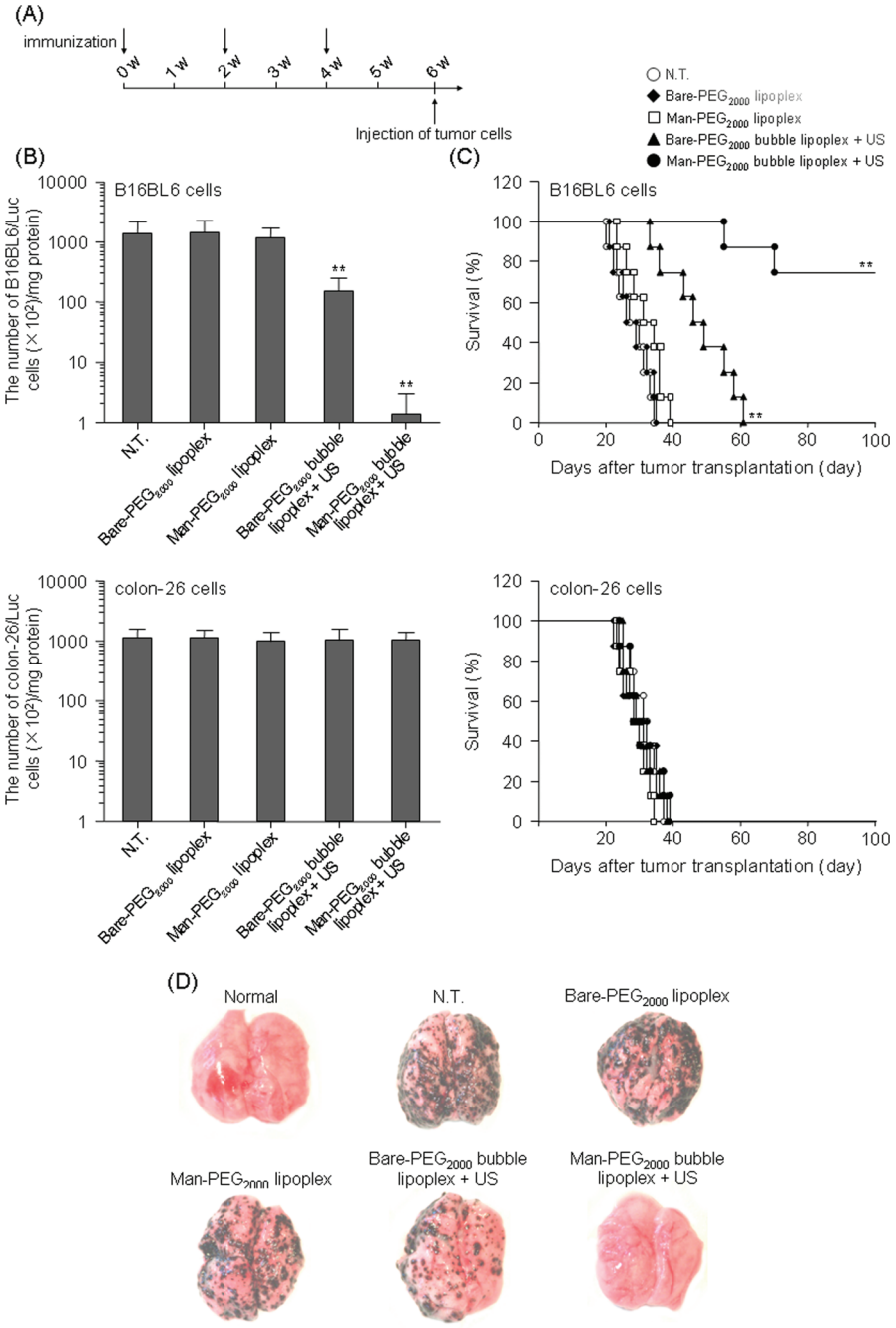

Figure 5. Cancer vaccine effects against pulmonary metastatic tumors by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments involving pulmonary metastatic tumors. (B, C) The suppressing effects of pulmonary metastatic tumors (B) and the prolonging of survival (C) by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA). Two weeks after the last immunization, B16BL6/Luc and colon-26/Luc cells (for the evaluation of tumor metastasis) and B16BL6 and colon-26 cells (for the evaluation of survival) were injected intravenously (1×10^5 cells) into mice. The pulmonary metastatic tumors at 14 days after the tumor injection were evaluated by the luciferase activity (n = 5, each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor injection (n = 8). $^{**}p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6-derived pulmonary metastatic tumor at 14 days after the tumor injection in mice immunized by each transfection method.

were sustained for at least 100 days against both solid and metastatic tumors.

DISCUSSION

The prognosis is poor for patients with melanoma, who exhibit a high rate of metastasis and relapse; therefore, the development of therapy for suppressing this melanoma metastasis and relapse is required.[2,3] It has been reported that DNA vaccination is effective for the prevention of metastasis and relapse,[5,7,8] and especially the application of DNA vaccination against melanoma has been focused since the identification of cancer antigens such as gp100, MART-1 and TRP is proceeding in melanoma.[10−13]

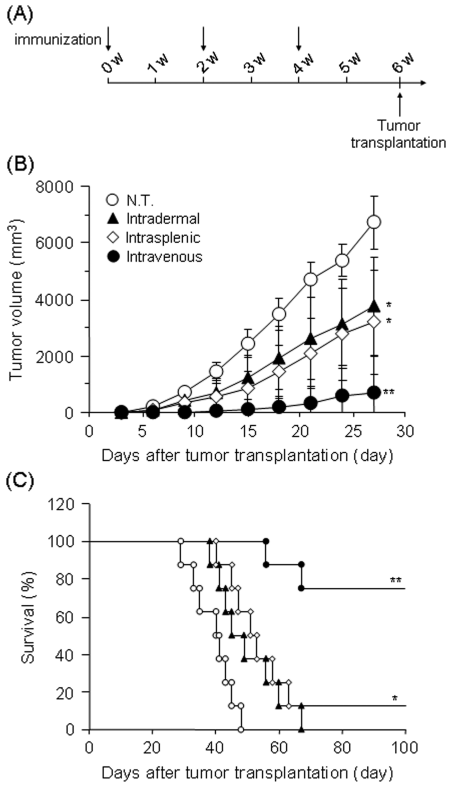

Figure 6. Effects of administration routes of Man-PEG$_{2000}$ bubble lipoplexes on DNA vaccine effects. (A) Schedule of therapeutic experiments. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging of survival in tumor-transplanted mice (C) by DNA vaccination using various administration routes of Man-PEG$_{2000}$ bubble lipoplexes (50 μg of pDNA) and US exposure. Man-PEG$_{2000}$ bubble lipoplexes were given by intradermal, intrasplenic and intravenous administration into mice, and US was exposed to the injected site directly or to the abdominal area externally. Two weeks after the last immunization, B16BL6 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8$). The tumor volume was evaluated (each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor transplantation. *$p < 0.05$; **$p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

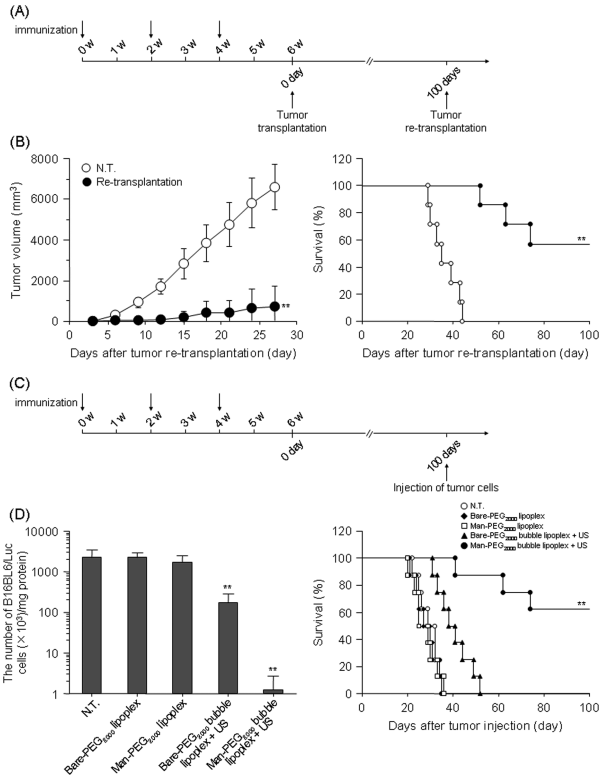

Figure 7. Duration of DNA vaccine effects by Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments against solid tumors. At 100 days after first transplantation of B16BL6 cells into mice immunized three times by Man-PEG$_{2000}$ bubble lipoplexes and US exposure, B16BL6 cells (1×10^6 cells) were retransplanted subcutaneously into the back of mice who completely rejected the first transplanted tumors ($n = 7$). (B) The suppressing effects of tumor growth against solid tumors and the prolonging of survival in tumor-transplanted mice by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure (50 μg of pDNA). The tumor volume was evaluated (each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor retransplantation. (C) Schedule of therapeutic experiments against metastatic tumors. At 100 days after the last immunization using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA), B16BL6 cells (for the evaluation of tumor metastasis) and B16BL6 cells (for the evaluation of survival days) were injected intravenously (1×10^5 cells) into mice. (D) The suppressing effects of B16BL6 cell-derived pulmonary metastatic tumors and the prolonging of survival by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA). The pulmonary metastatic tumors at 14 days after the injection of B16BL6 cells were evaluated by the luciferase activity ($n = 4$), and the survival was monitored up to 100 days ($n = 8$). **$p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

On the other hand, it is essential to transfer effectively into APCs such as dendritic cells to obtain potent therapeutic effects by DNA vaccination.[14,15] In the present study, we applied an APC-selective and -efficient gene transfection method using Man-PEG$_{2000}$ bubble lipoplexes constructed with gp100 and TRP-2-encoding pDNA and US exposure to DNA vaccination against melanoma with metastatic and relapsed properties.

The delivery of antigen-encoding gene into the dendritic cells, known as a major target cells for cancer immunotherapy, is necessary to achieve potent therapeutic effects with DNA vaccination.[14,15] However, it seems that the number of dendritic cells distributed in organs, such as spleen and skin, is low for DNA vaccination.[38] Moreover, gene transfection efficiency in dendritic cells is low,[20] because dendritic cells are poorly dividing cells[39,40] and immune effector cells are highly sensitive to cationic lipids.[41] To overcome these obstacles, gene transfection methods using external physical stimulation, such as electropolation, hydrodynamic injection and sonoporation, have been investigated for cancer vaccination.[21−25] In particular, sonoporation methods using microbubbles and US exposure are expected to be suitable as a gene transfection method for DNA vaccination in clinical situation, because microbubbles and US exposure systems have been used for diagnostic imaging[42,43] and calculus fragmentation[44,45] in clinical situation. We have developed a gene transfection method using Man-PEG$_{2000}$ bubble lipoplexes and US exposure, and succeeded in obtaining APC-selective and -efficient gene expression following experiments using luciferase-encoding pDNA.[33] In this study using pUb-M which expresses melanoma-related antigens (gp100 and TRP-2), a high level of expression in

the spleen was obtained by gene transfer using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figures 1A and 1B). Moreover, this gene expression was obtained selectively in the splenic $CD11c^+$ cells, known as dendritic cells[46] (Figure 1C), and these findings corresponded to those in our previous reports of using firefly luciferase-encoding pDNA.[33] In our previous report using pCMV-Luc and pCMV-OVA, we showed that the enhanced gene expression in the spleen was obtained by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure, and not observed in gene transfer using Man-PEG_{2000} lipoplexes or Man-PEG_{2000} bubble lipoplexes only and Man-PEG_{2000} lipoplexes with US exposure.[33] These observations suggest that splenic dendritic cell-selective and -efficient expression of melanoma-related antigens can be specifically obtained by the gene transfer using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure.

To achieve potent therapeutic effects by DNA vaccination against cancer, the activation of Th1 immunity and the effective induction of CTLs with high antitumor activities are important.[47] The antigen presentation on MHC class I molecules is essential for efficient CTL induction.[6,8,9] Antigens function as endogenous antigens since the cancer antigens are expressed intracellularly in DNA vaccination; consequently, the antigens are presented on MHC class I molecules.[8] As shown in Figure 2, the enhanced secretion of Th1 cytokines (IFN-γ and TNF-α) was observed in the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure by addition of B16BL6 cell lysates, compared with that of Th2 cytokines (IL-4 and IL-6). Moreover, the effective induction of CTLs against B16BL6 cells was also observed by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 3). Recently, we have reported that the antigen presentation on MHC class I molecules was also observed in DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with OVA-encoding pDNA.[33] These results suggest that antigen presentation of melanoma antigens on MHC class I molecules is responsible for the enhanced secretion of Th1 cytokines stimulated by B16BL6 cell and the induction of CTLs against B16BL6 cells in this study. As shown in Figure 4, the growth of B16BL6 cell-derived tumors was suppressed and the survival of B16BL6 cell-transplanted mice was prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Since the prognosis of patients with melanoma is poor, because of the high metastatic properties of melanoma as mentioned above,[2,3] we also investigated the vaccine effects against metastatic melanoma by DNA vaccination using this method. As shown in Figure 5, B16BL6 cell-derived pulmonary metastasis constructed by intravenous injection of tumor cells was suppressed by DNA vaccination using this gene transfection method. These DNA vaccine effects followed by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure were obtained against not only B16BL6 cells but also B16F1-derived tumors (Supplementary Figure 4 in the Supporting Information), suggesting that this DNA vaccination might be potent against various types of melanoma. On the other hand, the potent therapeutic effects against B16BL6-derived solid and metastatic tumor transplanted mice were not observed in DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure (data not shown). These findings suggest that the optimized duration for immunization is essential for obtaining potent antitumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure, and DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure may be suitable for the prevention of cancer metastasis and relapse. In addition, these vaccine effects against solid and metastatic tumors were sustained for at least 100 days (Figure 7). These observations lead us to believe that the enhanced secretion of Th1 cytokines and the induction of B16BL6 cell-specific CTLs contribute to the effective and long-term DNA vaccine effects against solid and metastatic tumors, following pUb-M transfer into splenic dendritic cells using Man-PEG_{2000} bubble lipoplexes and US exposure.

Intradermal and intrasplenic routes are widely used to transfer pDNA into the Langerhans cells known as dendritic cells in the skin or splenic dendritic cells.[48,49] On the other hand, we obtained potent therapeutic effects by DNA vaccination using intravenous administration of Man-PEG_{2000} bubble lipoplexes and external US exposure in this study. As shown in Figure 6, the DNA vaccine effects obtained by immunization using intravenous administration of Man-PEG_{2000} bubble lipoplexes were higher, compared with that using intradermal and intrasplenic administration. In the gene transfer using intradermal/intrasplenic administration of Man-PEG_{2000} bubble lipoplexes, it is assumed that the diffusibility of Man-PEG_{2000} bubble lipoplexes is not good and the delivering efficiency to the dendritic cells may be low, because of the large particle size of Man-PEG_{2000} bubble lipoplexes (approximately 500 nm (Table 1)). Therefore, it may be difficult to deliver the antigen-encoding pDNA into a large number of dendritic cells in the gene transfection process using intradermal/intrasplenic administration of Man-PEG_{2000} bubble lipoplexes. On the other hand, when Man-PEG_{2000} bubble lipoplexes were administered intravenously, the antigen-encoding pDNA may be delivered into a large number of dendritic cells widely distributed in the spleen through the blood vessels. Therefore, it is assumed that potent vaccine effects are obtained by gene transfer in the dendritic cells widely distributed in the spleen. These results suggest that the intravenous administration of Man-PEG_{2000} bubble lipoplexes is suitable to obtain high therapeutic effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure.

In this study, melanoma-specific vaccine effects were induced by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure, and moreover, intravenous administration of ManPEG_{2000} bubble lipoplexes was found to be suitable for DNA vaccination using this method in mice (Figure 6). For clinical application to achieve efficient DNA vaccination, Man-PEG_{2000} bubble lipoplexes need to be delivered to the spleen efficiently at a low dose. Recently, a medical catheter, which possesses a device to inject the microbubbles and to expose US, has been developed for the treatment of thrombolysis in clinical situation.[44,45] During treatment, this catheter is positioned within the lesion sites via the vessels, and various types of drugs, such as the lytic agents and microbubbles, are infused simultaneously with US exposure. Since this system may enable the local injection of Man-PEG_{2000} bubble lipoplexes and direct US exposure to the spleen by catheter delivery via the blood vessels, more potent DNA vaccine effects against melanoma are expected to be obtained at a low dose of Man-PEG_{2000} bubble lipoplexes by applying this catheter-based US system in the future.

CONCLUSION

In the present study, we developed DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M encoding ubiquitylated melanoma-specific antigens (gp100 and TRP-2) and US exposure, and succeeded in obtaining potent DNA vaccine effects against solid and metastatic cancers derived from B16BL6

melanoma specifically. Moreover, its vaccine effects against melanoma were sustained for 100 days at least. The findings obtained in this study suggest that the gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure could be suitable for DNA vaccination aimed at the prevention of metastatic and relapsed cancer.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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